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# Neuropeptide-degrading endopeptidase activity of locust (*Schistocerca gregaria*) synaptic membranes

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Locust adipokinetic hormone (AKH, pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-$NH_2$) was used as the substrate to measure neuropeptide-degrading endopeptidase activity in neutral membranes from ganglia of the locust *Schistocerca gregaria*. Initial hydrolysis of AKH at neural pH by peptidases of washed neural membranes generated pGlu-Leu-Asn and Phe-Thr-Pro-Asn-Trp-Gly-Thr-$NH_2$ as primary metabolites, demonstrating that degradation was initiated by cleavage of the Asn–Phe bond. Amastatin protected the *C*-terminal fragment from further metabolism by aminopeptidase activity without inhibiting AKH degradation. The same fragments were generated on incubation of AKH with purified pig kidney endopeptidase 24.11, an enzyme known to cleave peptide bonds that involve the amino group of hydrophobic amino acids. Phosphoramidon (10 $\mu$M), a selective inhibitor of mammalian endopeptidase 24.11, partially inhibited the endopeptidase activity of locust neural membranes. This phosphoramidon-sensitive activity was shown to be enriched in a synaptic membrane preparation with around 80% of the activity being inhibited by 10 $\mu$M-phosphoramidon ($IC_{50}$ = 0.2 $\mu$M). The synaptic endopeptidase was also inhibited by 1 mM-EDTA, 1 mM-1,10-phenanthroline and 1 $\mu$M-thiorphan, and the activity was maximal between pH 7.3 and 8.0. Localization of the phosphoramidon-sensitive enzyme in synaptic membranes is consistent with a physiological role for this endopeptidase in the metabolism of insect peptides at the synapse.

## INTRODUCTION

Although more than one mechanism may be involved in the termination of neuropeptide signals, studies on vertebrate systems indicate that peptide degradation may have an important role in the inactivation process (Kreiger, 1983; Mckelvy & Blumberg, 1986). Many of the enzymes considered to be involved in peptide inactivation are ectoenzymes, i.e. peptidases integrally bound to the plasma membrane and facing the extracellular space (Turner *et al.*, 1985; Kenny *et al.*, 1987).

In insects, comparatively little is known about the degradation of neuropeptides by plasma membrane enzymes from nervous tissue. A synaptic membrane preparation from locust ganglia is enriched with an aminopeptidase activity against the insect neuropeptide proctolin (Isaac, 1987). Unlike proctolin, many of the neuropeptides isolated from insects possess a pyroglutamyl (pGlu) residue at the *N*-terminus and have an amidated *C*-terminal [e.g. the adipokinetic hormone (AKH) family of insect neuropeptides (Orchard, 1987; Gade & Rinehart, 1986, 1987) and members of the leucokinin family (Holman *et al.*, 1986*a*,*b*, 1987*a*,*b*; Nachman *et al.*, 1986*a*,*b*)], which will confer protection from degradation by both amino- and carboxy-peptidase enzymes. The inactivation of peptides with blocked termini may therefore depend upon endopeptidases that can initiate degradation.

Synaptic membranes prepared from mammalian brain are enriched with an endopeptidase that has been implicated in the inactivation of peptides such as enkephalins (Schwartz *et al.*, 1981) and substance P (Turner *et al.*, 1985). The enzyme, endopeptidase 24.11, is a neutral metallopeptidase that can degrade a wide range of neuropeptides primarily through the hydrolysis of peptide bonds involving the amino group of hydrophobic amino acids (Matsas *et al.*, 1983, 1984; Turner, 1987). The enzyme has a wide tissue distribution and is selectively inhibited by phosphoramidon (Kenny, 1977). Although the physiological functions of this enzyme have not been precisely defined, its broad substrate specificity and tissue distribution suggest a possible general role for endopeptidase 24.11 in peptide inactivation (Turner *et al.*, 1985, 1987; Turner, 1987).

There have been no comparable studies to determine whether a similar endopeptidase capable of degrading peptides with blocked end groups is present in insect plasma membranes. In the present work, locust AKH (pGlu-Leu-Asn-Phe-Ser-Pro-Asn-Trp-Gly-Thr-$NH_2$), a peptide first isolated from *Schistocerca gregaria* by Stone *et al.* (1976), was used as the substrate for measuring endopeptidase activity in locust nervous tissue. AKH-like peptides have been localized in a number of interneurons of the insect central nervous system, where they may function as transmitter molecules (Schooneveld *et al.*, 1983, 1985; Schooneveld, 1986). AKH and the closely related peptides MI and MII, isolated from the cockroach *Periplaneta americana*, can cause contractions of insect muscle (O'Shea *et al.*, 1984; Scarborough *et al.*, 1984; Evans & Myers, 1986). Thus a number of peptides

Abbreviations used: AKH, adipokinetic hormone; pGlu, pyroglutamyl; $IC_{50}$, concn. causing 50% inhibition.

belonging to the AKH family are likely to have transmitter roles in addition to their hormonal functions of controlling lipid and carbohydrate metabolism (Orchard, 1987).

The present paper describes some properties of the endopeptidase activity of neural membranes from the locust *Schistocerca gregaria* using AKH as the substrate. The localization in insect synaptic membranes of a peptidase with properties similar to that of mammalian endopeptidase 24.11 is reported for the first time.

## MATERIALS AND METHODS

AKH was obtained from Peninsula Laboratories Europe, St. Helens, Merseyside, U.K. Amastatin, phosphoramidon, 1,10-phenanthroline, amino acids, dialysed Ficoll and sequencing-grade phenyl isothiocyanate were from Sigma Chemical Co., Poole, Dorset, U.K. H.p.l.c.-grade solvents were supplied by Rathburn Chemicals, Walkerburn, Scotland, U.K. 'Puriss'-grade HCl (Fluka Chemicals, Glossop, Derbyshire, U.K.) was used for peptide hydrolysis, and all other reagents were of AnalaR grade (BDH, Poole, Dorset, U.K.). Endopeptidase 24.11 purified from pig kidney was a generous gift from Dr. A. J. Kenny, Department of Biochemistry, University of Leeds, Leeds, U.K. Thiorphan was kindly provided by Professor B. P. Roques, Department de Chimie Organique, U 266 Institute de la Sante et de la Recherche Medicale, Faculte de Pharmacie, Paris, France.

### Enzyme preparations

Ganglia excised from male and female locusts were homogenized in 50 mM-Tris/HCl buffer, pH 7.3 (0.1 g of tissue/ml of buffer). The supernatant obtained from a low-speed (1000 *g* for 10 min) spin of the homogenate was centrifuged at 30000 *g* for 20 min (Beckman J2-21 centrifuge; JA-21 rotor) to provide a membrane pellet. The pellet was washed by resuspending in 0.5 M-NaCl/50 mM-Tris/HCl, pH 7.3, and centrifuging again at 30000 *g* for 20 min. The washed membrane pellet (P1) was resuspended in 10 mM-Tris/HCl, pH 7.3. Synaptosomal and mitochondrial membrane fractions were prepared as described previously (Isaac, 1987). Protein was estimated by the method described by Markwell *et al.* (1978).

### Enzyme assays

Assays for measuring the degradation of AKH routinely contained 100 $\mu$M-amastatin, 100 $\mu$M-AKH, 0.1 M-Tris/HCl, pH 7.3, and 1–2 $\mu$g of membrane protein. Reactions were allowed to proceed for up to 2 h at 30 °C. The same conditions were used to study the hydrolysis of AKH by porcine endopeptidase 24.11 (7.5 ng), except that the incubation time was reduced to 15 min and amastatin was omitted from the assay. Reactions were routinely started by the addition of substrate and terminated by the addition of 8 % (w/v) trichloroacetic acid (5 $\mu$l). For the inhibition experiments, enzyme preparations were preincubated with inhibitor for 10 min at 20 °C before the addition of AKH. Hydrolysis products were resolved and quantified by h.p.l.c. using a 3 $\mu$m Ultrasphere ODS column (75 mm × 4.6 mm; Beckman), a flow rate of 1 ml/min and a linear gradient of acetonitrile in 0.1 % (v/v) phosphoric acid increasing from 5 to 45 % in 8 min. Elution with 45 % acetonitrile in 0.1 % (v/v) phosphoric acid was maintained for a further 2 min. A u.v. detector set at either 214 or 280 nm was used to monitor the elution of peptide fragments. For the endopeptidase assay, the tryptophan-containing fragment and unhydrolysed AKH were quantified with the u.v. detector set at 280 nm.

### Amino acid analysis

Peptide samples were hydrolysed in 6 M-HCl under vacuum for 17 h at 110 °C. Amino acids were derivatized with phenyl isothiocyanate by using the procedure described by Bidlingmeyer *et al.* (1984). The phenylthiocarbamyl amino acids were resolved and quantified by h.p.l.c. using a 250 mm × 4.5 mm 5 $\mu$m ODS Hypersil column (Shandon Southern, Runcorn, Cheshire, U.K.) and the solvent system described by Ebert (1986). The recoveries of amino acids after hydrolysis and derivatization were obtained from the analysis of standard amino acids and authentic AKH.

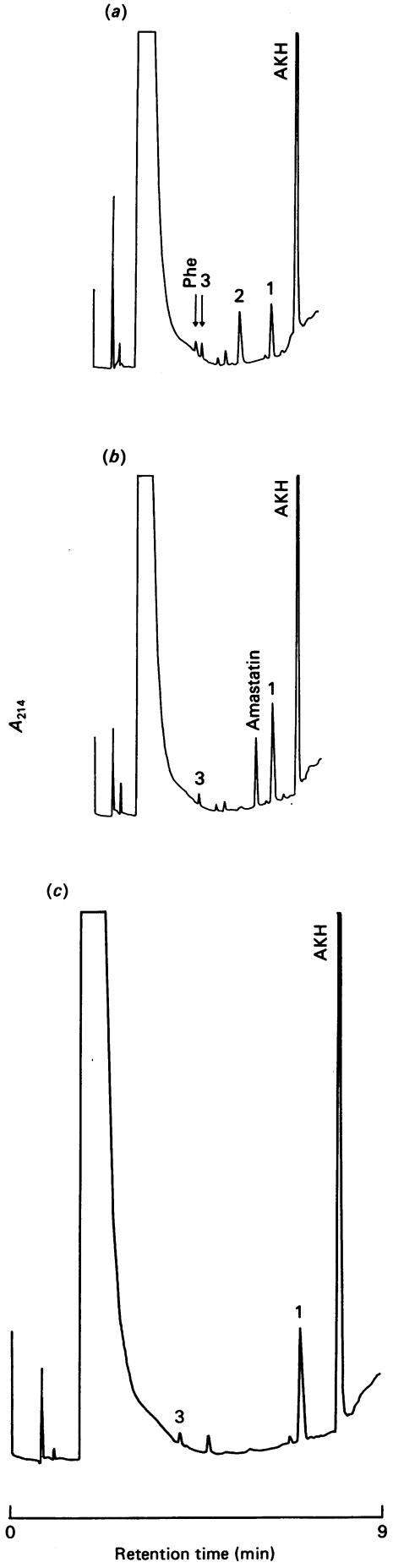
## RESULTS

### AKH degradation by neural membranes

Neural membranes (P1) were able to degrade AKH (100 $\mu$M) at pH 7.3 to two major u.v. (214 nm)-absorbing fragments (peaks 1 and 2) that were resolved by reversed-phase h.p.l.c. (Fig. 1*a*). Both products were also detected at 280 nm, demonstrating the presence of a tryptophan in both peptide fragments. A study of the time course for the hydrolysis of AKH suggested that peak 1 was an initial breakdown product, which was subsequently metabolized to peak 2 (Fig. 2*a*). The addition of the aminopeptidase inhibitor amastatin (100 $\mu$M) prevented the formation of peak 2 without inhibiting the degradation of AKH (Figs. 1*b* and 2*b*). Furthermore, amastatin inhibited the production of a small peak with an identical retention time to phenylalanine. Amino acid analysis (molar ratios are indicated in parentheses) of peak 1 showed that this fragment contained Phe (0.9), Pro (1.3), Asp (1.2), Gly (1.1) and Thr (2.00), in addition to tryptophan (absorbance at 280 nm) and identified peak 1 as Phe-Thr-Pro-Asn-Trp-Gly-Thr-$NH_2$, suggesting that the Asn–Phe bond was the initial site of AKH breakdown. A weak u.v. (214 nm)-absorbing fragment (peak 3) was isolated and identified as pGlu-Leu-Asn from the results of amino acid analysis [Asp (1.0), Glu (0.8), Leu (1.0)]. These same terminal fragments were also identified as hydrolysis products when AKH was incubated with purified endopeptidase 24.11 under initial hydrolysis conditions (Fig. 1*c*). These results confirm that the Asn–Phe bond is the site of initial hydrolysis of AKH by peptidases from locust neural membranes. This insect endopeptidase activity was partially inhibited (30 %) by 10 $\mu$M-phosphoramidon (Fig. 3), a concentration at which phosphoramidon selectively abolishes the activity of mammalian endopeptidase 24.11 (Matsas *et al.*, 1984).

### Subcellular localization

The crude neural (P1) membranes were separated into synaptic and mitochondrial membrane fractions and the ability of both preparations to degrade AKH, in the presence of amastatin (100 $\mu$M), was investigated. Both preparations yielded Phe-Thr-Pro-Asn-Trp-Gly-Thr-$NH_2$ as the only major product absorbing at both 214 and 280 nm. There was no significant enrichment of the peptidase activity in synaptic membranes



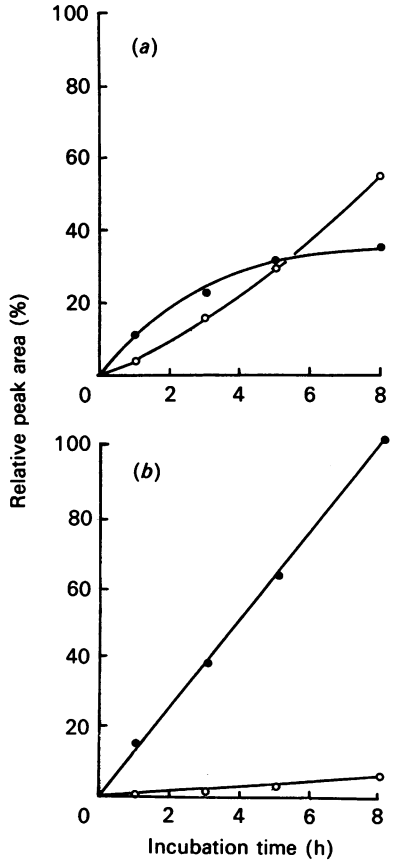




**Fig. 2. Time course for AKH metabolism**

Peptide fragments [peak 1, Phe-Thr-Pro-Asn-Trp-Gly-Thr-$NH_2$, (●); peak 2 (○)], produced on incubation of 100 $\mu$M-AKH with (P1) membranes in the absence (*a*) or presence (*b*) of 100 $\mu$M-amastatin, over an 8 h incubation period were quantified by reversed-phase h.p.l.c. See the Materials and methods section for details.

(1.20 ± 0.063 nmol/min per mg of protein; ± S.E.M.; $n = 3$) compared with the activity of P1 (1.01 ± 0.063 nmol/min per mg of protein; ± S.E.M.; $n = 3$) and mitochondrial (0.88 ± 0.075 nmol/min per mg of protein; ± S.E.M.; $n = 3$) membrane preparations.

Inhibition curves for phosphoramidon were also obtained using synaptic and mitochondrial membrane fractions as the source of AKH-degrading activity (Fig. 3). It was clear that the bulk (> 60 %) of the phosphoramidon-sensitive activity detected in crude P1 membranes was localized in the synaptic membrane preparation, whilst over 80 % of the endopeptidase activity in the mitochondrial fraction was not inhibited, even at higher phosphoramidon concentrations (10–100 $\mu$M). By using

**Fig. 1. AKH hydrolysis by locust neural membranes (P1) and endopeptidase 24.11**

AKH (100 $\mu$M) was incubated with neural membranes (P1) (*a* and *b*) or purified endopeptidase-24.11 from pig kidney (*c*). In experiment (*b*) the membrane preparation was preincubated with amastatin (100 $\mu$M, final concn.). Peptide fragments (peak 1, Phe-Thr-Pro-Asn-Trp-Gly-Thr-$NH_2$; peak 2, not analysed; peak 3, pGlu-Leu-Asn) were resolved by reversed-phase h.p.l.c. See the Materials and methods section for details.

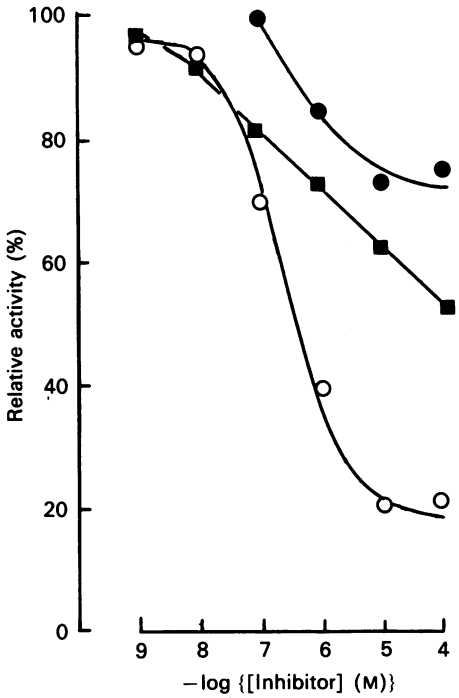



**Fig. 3. Inhibition of AKH metabolism by phosphoramidon**

Inhibition of AKH hydrolysis by endopeptidase activity of P1 (■) synaptic (○) and mitochondrial (●) membrane fractions from locust by phosphoramidon. Experimental details are given in the text.

these data, it was estimated that the phosphoramidon-sensitive enzyme was enriched around 3-fold in the synaptic membrane preparation compared with P1 membranes.

### Properties of the synaptic endopeptides

Phosphoramidon inhibited the synaptic membrane activity with an $IC_{50}$ of 200 nM. Thiorphan also selectively inhibited 60% of the activity at a concentration of 1 μM. AKH hydrolysis, measured in the presence of 100 μM-amastatin, was maximal over a pH range of 7.3 to 8.1 and was inhibited by 1 mM-EDTA and 1 mM-1,10-phenanthroline (40 and 100% inhibition, respectively).

## DISCUSSION

The results of the present work indicate that neural membranes from locust ganglia are able to degrade locust AKH, primarily through hydrolysis of the Asn–Phe bond to release pGlu-Leu-Asn and Phe-Thr-Pro-Asn-Trp-Gly-Thr-$NH_2$. The *C*-terminal fragment, Phe-Thr-Pro-Asn-Trp-Gly-Thr-$NH_2$ was protected from further metabolism by the aminopeptidase inhibitor amastatin and was quantified by h.p.l.c. to provide a convenient and sensitive assay of the insect endopeptidase. The heptapeptide was also shown to be the major tryptophan-containing fragment generated on incubation of AKH with purified endopeptidase 24.11 from pig kidney. This result is consistent with the known hydrolytic properties of the mammalian enzyme, which shows a preference for peptide bonds that involve the amino acid of hydrophobic amino acids (e.g. Phe, Leu, Trp, Tyr) and in particular, phenylalanine (Matsas *et al.*, 1984*a*,*b*; Stephenson & Kenny, 1987*a*,*b*). It was therefore predicted that cleavage of the Asn–Phe bond of AKH would be the primary cleavage site for endopeptidase 24.11.

Phosphoramidon inhibited a great proportion of the locust synaptic endopeptidase activity, but not the activity associated with a mitochondrial membrane preparation. This inhibitor is a natural product that selectively inhibits endopeptidase 24.11 ($IC_{50}$, 2 nM; Kenny, 1977; Fulcher *et al.*, 1982) and thermolysin (Suda *et al.*, 1973), both enzymes being Zn-metalloendopeptidases. Thiorphan, another potent inhibitor of mammalian endopeptidase 24.11 ($K_i$ = 3.5 nM; Roques *et al.*, 1980), also inhibited the insect endopeptidase at a concentration of 1 μM. This inhibitor displays lower selectivity compared with phosphoramidon, as it also inhibits mammalian peptidyl-dipeptidase A (angiotensin converting enzyme EC 3.4.15.1), at higher concentrations (0.1–1 μM; Roques *et al.*, 1980; Matsas *et al.*, 1984). The lower potency of both phosphoramidon and thiorphan on the locust endopeptidase probably reflects an important difference between the substrate-binding sites of the insect and mammalian enzyme. Any enzymic metabolism of the inhibitors by the crude enzyme preparation will also lower the effectiveness of these compounds on the insect enzyme. The inhibition of the insect synaptic endopeptidase activity by EDTA (1 mM) and 1,10-phenanthroline (1 mM) is consistent with a requirement for metal ions. Studies on the phosphoramidon-insensitive endopeptidase localized in the mitochondrial fraction have not as yet been pursued.

The locust synaptic enzyme appears to have a similar substrate specificity to that of endopeptidase-24.11 and may, therefore, have an important role in the metabolism of an array of insect neuropeptides that possess internal hydrophobic residues. Of the insect neuropeptides isolated to date, many belong to the AKH and leucokinin families, and all members of both groups possess phenylalanine at position 4 (Orchard, 1987; Holman *et al.*, 1986*a*,*b*, 1987*a*,*b*; Gade & Rinehart, 1986, 1987). Whether the phosphoramidon-sensitive endopeptidase is involved in the metabolism of these various insect neuropeptides remains to be established.

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